ANTIPARASITIC TREATMENT SUPPRESSES PRODUCTION AND AVIDITY OF *TOXOPLASMA GONDII*-SPECIFIC ANTIBODIES IN A MURINE MODEL OF ACUTE INFECTION*

C. Alvarado-Esquivel¹, A. Niewiadomski, B. Schweickert², O. Liesenfeld^{3**}

Department of Microbiology and Hygiene, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

¹Present address: Departamento de Infectología, Facultad de Medicina y Nutrición, Universidad Juárez del Estado de Durango, Durango, Dgo. México, Avenida Universidad y Fanny Anitua, 34000 Durango, Dgo. Mexico

²Present address: Robert-Koch-Institut, Nordufer 20, D-13353 Berlin, Germany

³Present address: Medical and Scientific Affairs, Roche Molecular Diagnostics, 4300 Hacienda Dr., Pleasanton, CA 94588, USA

Received: July 8, 2011; Accepted: July 14, 2011

Infection with *Toxoplasma gondii* during pregnancy may result in congenital transmission of the parasite. Infection is commonly diagnosed using serological tests for IgG, IgM and IgA antibodies. Avidity of IgG antibodies is used to exclude acute infection. Few studies have investigated the impact of antiparasitic treatment on the production of anti-*T. gondii* antibody and the avidity of IgG antibodies. We therefore investigated the production of IgG, IgM, and IgA antibodies and IgG avidity in a murine model of acute infection with 10 cysts of *T. gondii*. All antibody classes increased following infection. Treatment of mice with pyrimethamine/sulfadiazine but not with spiramycin or azithromycin at dosages equivalent to those used in patients resulted in a significant decrease in the concentration of *T. gondii*-specific IgG and IgM antibodies postinfection. IgG and IgM antibody decreases were paralleled by a significant reduction in cyst numbers in brains of mice treated with pyrimethamine/sulfadiazine but not with other drugs. In contrast, treatment with atovaquone did significantly reduce the concentrations of IgM antibodies and resulted in reduced IgG avidity indices. *T. gondii*-specific DNA was not detected in blood between days 1 and 3. In conclusion, antiparasitic treatment with pyrimethamine/sulfadiazine and atovaquone appears to impact the generation of antibody responses against *T. gondii*. Future studies will have to determine the specific impact of antiparasitic treatment on antibody responses and the consequences for the management of patients infected with *T. gondii*.

Keywords: antibodies, IgG avidity, *Toxoplasma gondii* infection, treatment, mice

Introduction

It has been estimated that about one-third of the human population is infected with the protozoan parasite *Toxoplasma gondii* [1]. Infection with *T. gondii* is usually asymptomatic. However, some *T. gondii*-infected patients develop cervical lymphadenopathy and/or chorioretinitis. Importantly, acute infection in pregnant women may result in congenital disease in the fetus [1]. Clinical manifestations of congenital toxoplasmosis include chorioretinitis, strabismus, blindness, hydrocephalus, microcephaly, intracranial calcification, epilepsy and psychomotor or mental retardation [1–3]. The risk of mother-to-child transmission of *T. gondii* was shown to be inversely proportional to the stage of pregnancy at which maternal infection occurs [4–6]. Thus, dating of maternal *Toxoplasma* infection during pregnancy is of utmost importance

to determine the risk of fetal infection. Serological tests for measurement of IgG, IgM, IgA and IgE antibodies have been successfully used as a panel of serological tests [7, 8]. In pregnant women, a combination of these antibody titers and the IgG avidity index is used to estimate the time that has elapsed since infection [9–12]. However, a number of factors, marked heterogeneity of antibody responses, gestational age, and antiparasitic treatment among others, may impact the production of *T. gondii*-specific antibodies and IgG avidity maturation. In this regard, only a limited number of studies on the effect of treatment on the generation of antibody responses have been published, with contradictory findings [11, 13–17].

We therefore investigated the production of *T. gondii*-specific IgG, IgM and IgA antibodies in mice orally infected with 10 cysts of *T. gondii* and treated with antiparasitic drugs used for the prevention and treatment of infection in humans.

*Parts of this work were presented at ECCMID 2008 (abstract P1875).

**Corresponding author: Oliver Liesenfeld; Department of Microbiology and Hygiene, Charité Campus Benjamin Franklin, Hindenburgdamm 27, D-12203 Berlin, Germany; Phone: +493084453628; Fax: +493084453830; E-mail: oliver.liesenfeld@charite.de

Materials and methods

Mice and T. gondii infection

Twelve-week-old female BALB/c mice were perorally infected with 10 *T. gondii* cysts of the ME49 strain. Cysts were maintained in the laboratory by passage in Swiss Webster mice-infected i.p. with 10 cysts every 3–6 months.

Toxoplasma gondii antigen

T. gondii lysate antigen (TLA) used in the enzyme immunoassays for determination of anti-*T. gondii* IgG, IgM, IgA and IgG avidity was prepared from the virulent BK strain of *T. gondii*. BALB/c mice were infected with 10^6 *T. gondii* tachyzoites, and tachyzoites were obtained from the peritoneal cavities 3 days later. Peritoneal fluid containing tachyzoites was sonicated and stored at -20°C until use.

Antiparasitic drug treatment

Concentrations of five antiparasitic drugs used to prevent or treat infection with *T. gondii* and the duration of treatment with these drugs were chosen to mimic antiparasitic treatment in patients infected with *T. gondii*. Spiramycin (Rovamycin[®], Teofarma srl. Valle Salimbene-Pavia, Italy) was administered at 0.86 mg per mouse/day. Pyrimethamine (Sigma Chemical Co. St. Louis, MO, USA) and sulfadiazine (Sigma) were administered at 0.06 mg per mouse/day and 2 mg per mouse/day, respectively. Azithromycin (Pfizer GmbH. Karlsruhe, Germany) was administered at 0.43 mg per mouse/day, and atovaquone (Wellvone[®], Glaxo Wellcome GmbH & Co. Bad Oldesloe, Germany) was administered at 0.86 mg per mouse/day. All drugs were administered by gavages once a day during 7 consecutive days starting at day 4 after infection.

Sampling of blood

Blood samples were obtained from uninfected control and antiparasitic drug-treated mice at 14, 25 and 60 days postinfection. Mice were anesthetized and blood was obtained by cardiac puncture. Serum was obtained by centrifugation of blood samples for 5 min at $> 10,000$ rpm.

Anti-T. gondii IgG ELISA and avidity test

Serum IgG anti-*T. gondii* antibody concentrations and their avidity were measured by enzyme-linked immuno-sorbent assay (ELISA) as follows: first, optimum assay conditions were determined by varying serum dilutions (1:1, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200), TLA concentrations (2.5, 5, 10, 20 and 40 $\mu\text{g/ml}$), and urea concentrations (4, 6 and 8 M) using sera from five uninfected control mice and eight

T. gondii-infected mice (obtained 2–54 weeks after infection). Serum dilution of 1:200, coating of plates with TLA concentration of 5 $\mu\text{g/ml}$, and urea concentrations of 6 M were found to yield optimum results. The cut-off value for optical density (OD) was fixed at the mean plus 3 standard deviations of absorbance in normal controls: 1.3. ODs in uninfected mice were < 0.1 . Anti-*T. gondii* IgG was detectable in all sera 3 weeks postinfection. The intra-assay variance of anti-*T. gondii* IgG ELISA and avidity test was determined using five replicates of serum samples obtained at different time-points of infection (0, 2, 3, 14 (2 independent samples) and 54 (2 independent samples) weeks). The inter-assay precision was determined using three independent assays and five replicates of the same set of sera described above. The intra- and inter-assay coefficients of variation were $< 15\%$.

The final protocol was as follows: 96-flat-bottom-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with TLA at a final concentration of 5 $\mu\text{g/ml}$ in 50-mM sodium carbonate buffer (pH 9.6). The plates were then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T20) (pH 7.4). The plates were blocked with PBS containing 4% bovine serum albumin (BSA) (PBS-4%BSA) for 1 h at 37°C . Then 1/200 dilutions of serum samples in PBS (100 μl /well) were added to duplicated wells and incubated for 1 h at 37°C . After washing the plates three times, PBS-T20 was added to one row of the duplicated wells, and 6-M urea-PBS-T20 was added to the second row of the duplicated wells. After incubation for 30 min at 37°C , the plates were washed once again with PBS-T20. One hundred microliters of alkaline phosphatase-conjugated goat anti-mouse IgG antibody (clone M30108, Caltag Laboratories, Burlingame, CA) diluted 1:2000 in PBS-4%BSA were added to each well. After 2 h at 37°C , plates were washed three times with PBS-T20. Thereafter, plates were incubated for 30 min at room temperature with *p*-nitrophenylphosphate (Sigma Aldrich) at 1 mg/ml in 1-M diethanolamine buffer (pH 9.8). The reaction was stopped by adding 50 μl /well of 3-N NaOH, and absorbance was read at 405 nm. The avidity index was calculated by the following formula: percentage of urea-resistant IgG of total bound IgG ($A_{405\text{Urea}+}/A_{405\text{Urea}-}$). Only samples with OD above the cut-off value of 1.3 of total bound IgG were used for the determination of IgG avidity.

Anti-T. gondii IgM and IgA ELISA

Alkaline phosphatase-conjugated goat anti-*T. gondii* IgM (clone M31508, Caltag) and IgA (clone M31108, Caltag) antibodies were used to determine concentrations of anti-*T. gondii* IgM and IgA antibodies in sera of mice as described above for IgG antibodies.

Numbers of T. gondii cysts in brains of mice

Numbers of cysts in brains were determined by immunohistochemistry using the peroxidase–anti-peroxidase me-

thod as previously described [18]. Sagittal sections of mouse brains were stained with anti-*T. gondii* hyper-immune serum obtained from a rabbit infected with 10 cysts of *T. gondii* and boosted with i.p. RH tachyzoites of *T. gondii*.

T. gondii DNA in blood

Approximately 25 µl of blood was obtained from the tail vein. DNA was extracted using the Qiagen DNA Blood Mini kit (Qiagen, Hilden, Germany). Quantitative PCR targets the *T. gondii* cryptic gene [19] in 10-µl reaction volumes containing 250 ng of total DNA, 2-µl enzyme mix, 2-µM MgCl₂, 1 µM of oligonucleotide primers (TOX-9, 5'-AggAgAgATATCAgACTgTAg-3'; TOX-10as, 5'-gCgTCgTCTCgTCTAgATCg-3'), and 0.2 µM of each fluorescent hybridization probe (TOX-HP-1, 5'-GAGTCG-GAGAGGGAGAAGATGTT-6-carboxyfluorescein-3'; TOX-HP-2, 5'-CCGGCTTGGCTGCTTTTCCTG-3') (all TIB Molbiol, Berlin, Germany). Polymerase chain reaction (PCR) was performed as follows: 95 °C for 10 min, followed by 50 cycles at 95 °C for 10 s, 52 °C for 20 s, and 72 °C for 30 s. After a final extension step at 40 °C for 30 s, the samples were cooled and stored at 4 °C. With each run, a standard curve was performed using 250 pg to 2.5 pg *T. gondii* DNA of green-fluorescent protein (GFP)-expressing

tachyzoites; distilled water served as the negative control. Fluorescence was analyzed by LightCycler data analysis software 3.5 (Roche, Mannheim, Germany). Crossing points (Cp) were established using the second derivative method.

Statistical analysis

All experiments were repeated twice. Differences between mean values were analyzed by unpaired Student's *t*-test. *P* values less than 0.05 were considered significant. Statistical analysis was performed with InStat version 2.0 software (GraphPad, San Diego, USA).

Results

After infection, there was a significant increase in specific anti-*T. gondii* IgG in all mice (Fig. 1). However, mice treated with pyrimethamine/sulfadiazine but not those treated with spiramycin, azithromycin or atovaquone showed significantly lower IgG levels than untreated control mice at 14 and 60 days after infection.

Since IgG avidity is commonly used in pregnant women with IgG antibodies against *T. gondii* to determine the time of infection, we investigated the avidity of IgG antibodies over time after infection. Untreated control mice and mice

Fig. 1. Development of anti-*T. gondii* IgG antibodies in mice infected with 10 cysts of *T. gondii*. Values represent the mean absorbance $\pm$ SD detected in each group. IgG concentrations were significantly lower in mice treated with pyrimethamine/sulfadiazine at days 14 and 60 compared to untreated mice

Fig. 2. Maturation of anti-*T. gondii* IgG avidity in mice from day 14 until day 60 postinfection with 10 cysts of *T. gondii*. Values represent the mean avidity index determined in each group. The IgG avidity index was significantly lower in mice treated with atovaquone compared to untreated mice at day 60 postinfection

treated with pyrimethamine/ sulfadiazine, spiramycin and azithromycin showed a marked increase in IgG avidity indices from days 14 to 25 and 60 similar to that observed in untreated mice (Fig. 2). Compared to control mice, only mice treated with atovaquone had significantly decreased IgG avidity indices at 60 days postinfection.

IgM concentrations increased between days 0 and 14 after infection in all groups, and were maintained at similar levels until 60 days after infection (Fig. 3). Treatment with either pyrimethamine/sulfadiazine (d25) or atovaquone (days 14, 25 and 60) significantly decreased IgM concentration compared to that in untreated control mice.

Concentrations of IgA antibodies also increased over time (Fig. 4). Neither of the antiparasitic treatments decreased the concentrations of IgA antibodies significantly after infection.

T. gondii cysts were detectable in brains of mice in all groups after infection. Numbers of cysts in untreated mice at days 25 and 60 were 6.75 ± 2.75 and 3.75 ± 4.4 , respectively. Numbers of cysts in mice treated with pyrimethamine/sulfadiazine (undetectable at day 25 and 0.5 ± 0.58 at day 60) but not in those treated with spiramycin, azithromycin and atovaquone were significantly lower than those in untreated mice at days 25 and 60 postinfection (data not shown). We did not detect *T. gondii*-specific DNA in blood samples obtained between days 1 and 3 postinfection using real-time PCR.

Discussion

The decision on treatment of pregnant patients with suspected acute toxoplasmosis is often based on results of laboratory tests, that is, antibody titers and the avidity of IgG antibodies. Results of these tests allow one to distinguish between acute and latent infection and to determine the risk of congenital transmission of the parasite [1, 20, 21]. However, there is evidence from observational studies that antiparasitic treatment with spiramycin may impact the production and avidity of *T. gondii*-specific antibodies [16, 17]. In this study, we therefore investigated the impact of treatment with a number of antiparasitic drugs used for the prevention and treatment of infection with *T. gondii* in humans on the production and avidity of antibodies in a murine model of acute toxoplasmosis.

Interestingly, we detected an impact of only those drugs with the highest antiparasitic efficacy, pyrimethamine/sulfadiazine and atovaquone, on the production and maturation of *T. gondii*-specific antibodies. The combination of pyrimethamine/sulfadiazine decreased the concentration of *T. gondii*-specific IgG and IgM antibodies. Since the 1950s, the combination of pyrimethamine/sulfadiazine has been considered as the most effective drug combination [22]. This combination proved remarkably efficient in the treatment of toxoplasmosis, both in experimental models and in humans [23, 24]. In newborns, specific IgG titers and im-

Fig. 3. Development of anti-*T. gondii* IgM antibodies in mice infected with 10 cysts of *T. gondii*. Values represent the mean absorbance $\pm$ SD detected in each group. Concentrations of IgM antibodies were significantly lower in mice treated with pyrimethamine/sulfadiazine (day 25 p.i.) and those treated with atovaquone (days 14, 25 and 60)

Fig. 4. Development of anti-*T. gondii* IgA antibodies in mice infected with 10 cysts of *T. gondii*. Values represent the mean absorbance $\pm$ SD detected in each group

mune loads were diminished to become almost nonexistent at the end of the year of treatment with pyrimethamine/sulfadiazine [25].

Atovaquone did not significantly suppress the production of *T. gondii*-specific IgG antibodies itself but significantly delayed the maturation of IgG antibodies as indicated by reduced avidity indices. In addition, treatment with atovaquone decreased the concentrations of IgM antibodies after infection. Interestingly, neither pyrimethamine/sulfadiazine nor atovaquone significantly modified the production of IgA antibodies. This result could indicate that the effect of atovaquone is specific to certain antibody responses by a yet unknown mechanism, or the group sizes used in the current study did not allow the detection of significant differences in the concentrations of IgG antibodies. In murine models of acute toxoplasmosis, administration of atovaquone alone resulted in a significant protection of infected mice, which correlates a marked reduction of parasitic burdens in tissues [26, 27]. However, there appears to be variation in the activity of atovaquone against *T. gondii* in humans [28].

We did not detect any impact of treatment on antibody responses following treatment with the macrolides spiramycin or azithromycin. These results while in contrast to some reports in patients are in line with the poor antiparasitic activity of spiramycin and other macrolides in experimental studies [29–32], and confirm results of other studies that did not find an impact of antiparasitic treatment on avidity maturation [11, 13]. The activity of azithromycin on cerebral toxoplasmosis is limited by its poor brain penetration [30]; in murine models of chronic infection, only long-term administration of spiramycin or azithromycin resulted in a significant reduction of brain cysts burdens [29, 33].

To the best of our knowledge, the impact of antiparasitic treatment on antibody production and maturation of IgG avidity has only been investigated in patient cohorts but not experimentally. Sensini et al. [14] first hypothesized that the treatment slowed the maturation of avidity and compared the mean IgG avidity values between treated and untreated women; unfortunately, the type of treatment or its length was not specified. Lefevre-Pettazzoni et al. [16] reported a significant decrease in IgG avidity for each additional week of gestational age before infection, and an increase in IgG avidity for each additional week of delay to the onset of spiramycin treatment. More recently, Meroni et al. [17] reported that *T. gondii*-specific IgG antibodies and *T. gondii*-specific IgG avidity indices were significantly delayed in pregnant women receiving therapy compared to nonpregnant, untreated controls. In contrast, Jennum et al. [11] did not find significant differences in mean IgG avidities before and after treatment in pregnant women with *Toxoplasma* seroconversion. In addition, Flori et al. [13] compared the IgG avidities of four groups of women treated for different lengths of time and a control group, but similar to the previous finding, found no significant differences in antibody responses.

Since a number of factors impact the generation of antibody responses in humans, the impact of treatment on the

antibody response can only be investigated in experimental models of infection in a more controlled setting. The infection used in this study appears to be a suitable model. Acute infection in humans was mimicked by a low-dose oral infection of mice; the start of treatment after infection as well as the length and dose of treatment were aligned with the treatment in humans. The model proved suitable to determine IgG, IgM and IgA antibody concentrations, the avidity of IgG antibodies, as well as cyst numbers and *Toxoplasma* DNA in blood. However, the results have to be interpreted with caution. First, mice were not pregnant when infected, and therefore the effect of gestation on the production of antibodies and the maturation of IgG avidity [16] could not be investigated. The effect of gestational age at the time of seroconversion on the maturation of IgG avidity may be explained by the modification of T-cell immune response during pregnancy to avoid rejection of the fetus. CD4+ CD25+ regulatory T cells control the production and maturation of the affinity of antigen-specific antibodies, and they proliferate during the second and the third trimesters of pregnancy [34–36]. Second, numbers of mice in the experimental groups may have been too low to observe statistically significant differences between antibody and avidity levels following antiparasitic treatment.

In conclusion, the results of this study demonstrate that anti-*T. gondii* treatment with pyrimethamine/sulfadiazine and atovaquone modifies *T. gondii*-specific antibody responses in mice experimentally infected with *T. gondii*. Future studies will have to confirm these findings in patients acutely infected with *T. gondii*.

References

1. Montoya JG, Liesenfeld O: Toxoplasmosis. *Lancet* 363, 1965–1976 (2004)
2. Swisher CN, Boyer K, McLeod R: Congenital toxoplasmosis. The Toxoplasmosis Study Group. *Semin Pediatr Neurol* 1, 4–25 (1994)
3. McLeod R, Kieffer F, Sautter M, Hosten T, Pelloux H: Why prevent, diagnose and treat congenital toxoplasmosis? *Mem Inst Oswaldo Cruz* 104, 320–344 (2009)
4. Romand S, Wallon M, Franck J, Thulliez P, Peyron F, Dumon H: Prenatal diagnosis using polymerase chain reaction on amniotic fluid for congenital toxoplasmosis. *Obstet Gynecol* 97, 296–300 (2001)
5. Hohlfeld P, Daffos F, Costa JM, Thulliez P, Forestier F, Vidaud M: Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. *N Engl J Med* 331, 695–699 (1994)
6. Dunn D, Wallon M, Peyron F, Petersen E, Peckham C, Gilbert R: Mother-to-child transmission of toxoplasmosis: risk estimates for clinical counselling. *Lancet* 353, 1829–1833 (1999)
7. Liesenfeld O, Montoya JG, Tathinen NJ et al.: Confirmatory serologic testing for acute toxoplasmosis and rate of induced abortions among women reported to have positive *Toxoplasma* immunoglobulin M antibody titers. *Am J Obstet Gynecol* 184, 140–145 (2001)
8. Montoya JG: Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. *J Infect Dis* 185, S73–S82 (2002)

9. Hedman K, Lappalainen M, Seppä I, Makela O: Recent primary toxoplasma infection indicated by a low avidity of specific IgG. *J Infect Dis* 159, 736–740 (1989)
10. Liesenfeld O, Montoya JG, Kinney S, Press C, Remington JS: Effect of testing for IgG avidity in the diagnosis of *Toxoplasma gondii* infection in pregnant women: experience in a US reference laboratory. *J Infect Dis* 183, 1248–1253 (2001)
11. Jenum PA, Stray-Pedersen B, Gundersen AG: Improved diagnosis of primary *Toxoplasma gondii* infection in early pregnancy by determination of antitoxoplasma immunoglobulin G avidity. *J Clin Microbiol* 35, 1972–1977 (1997)
12. Lappalainen M, Koskela P, Koskiniemi M et al.: Toxoplasmosis acquired during pregnancy: improved serodiagnosis based on avidity of IgG. *J Infect Dis* 167, 691–697 (1993)
13. Flori P, Tardy L, Patural H et al.: Reliability of immunoglobulin G antitoxoplasma avidity test and effects of treatment on avidity indexes of infants and pregnant women. *Clin Diagn Lab Immunol* 11, 669–674 (2004)
14. Sensini A, Pascoli S, Marchetti D et al.: IgG avidity in the serodiagnosis of acute *Toxoplasma gondii* infection: a multicenter study. *Clin Microbiol Infect* 2, 25–29 (1996)
15. Petersen E, Borobio MV, Guy E et al.: European multicenter study of the LIAISON automated diagnostic system for determination of *Toxoplasma gondii*-specific immunoglobulin G (IgG) and IgM and the IgG avidity index. *J Clin Microbiol* 43, 1570–1574 (2005)
16. Lefevre-Pettazzoni M, Bissery A, Wallon M, Cozon G, Peyron F, Rabilloud M: Impact of spiramycin treatment and gestational age on maturation of *Toxoplasma gondii* immunoglobulin G avidity in pregnant women. *Clin Vaccine Immunol* 14, 239–243 (2007)
17. Meroni V, Genco F, Tinelli C et al.: Spiramycin treatment of *Toxoplasma gondii* infection in pregnant women impairs the production and the avidity maturation of *T. gondii*-specific immunoglobulin G antibodies. *Clin Vaccine Immunol* 16, 1517–1520 (2009)
18. Dunay IR, Heimesaat MM, Bushrab FN et al.: Atovaquone maintenance therapy prevents reactivation of toxoplasmic encephalitis in a murine model of reactivated toxoplasmosis. *Antimicrob Agents Chemother* 48, 4848–4854 (2004)
19. Reischl U, Bretagne S, Kruger D, Ernault P, Costa JM: Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis* 3, 7 (2003)
20. Montoya JG: Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. *J Infect Dis* 185(Suppl 1), S73–S82 (2002)
21. Montoya JG, Remington JS: Management of *Toxoplasma gondii* infection during pregnancy. *Clin Infect Dis* 47, 554–566 (2008)
22. Eyles DE, Coleman N: The effect of metabolites on the anti-toxoplasmic action of pyrimethamine and sulfadiazine. *Am J Trop Med Hyg* 9, 277–283 (1960)
23. Huskinson-Mark J, Araujo FG, Remington JS: Evaluation of the effect of drugs on the cyst form of *Toxoplasma gondii*. *J Infect Dis* 164, 170–171 (1991)
24. Nguyen BT, Stadtsbaeder S: Comparative effects of cotrimoxazole (trimethoprim-sulphamethoxazole), pyrimethamine-sulphadiazine and spiramycin during avirulent infection with *Toxoplasma gondii* (Beverly strain) in mice. *Br J Pharmacol* 79, 923–928 (1983)
25. Kieffer F, Thulliez P, Brezin A et al.: Treatment of subclinical congenital toxoplasmosis by sulfadiazine and pyrimethamine continuously during 1 year: apropos of 46 cases. *Arch Pediatr* 9, 7–13 (2002)
26. Araujo FG, Huskinson J, Remington JS: Remarkable *in vitro* and *in vivo* activities of the hydroxynaphthoquinone 566C80 against tachyzoites and tissue cysts of *Toxoplasma gondii*. *Antimicrob Agents Chemother* 35, 293–299 (1991)
27. Araujo FG, Huskinson-Mark J, Gutteridge WE, Remington JS: *In vitro* and *in vivo* activities of the hydroxynaphthoquinone 566C80 against the cyst form of *Toxoplasma gondii*. *Antimicrob Agents Chemother* 36, 326–330 (1992)
28. Kovacs JA: Efficacy of atovaquone in treatment of toxoplasmosis in patients with AIDS. The NIAID-Clinical Center Intramural AIDS Program. *Lancet* 340, 637–638 (1992)
29. Grujic J, Djurkovic-Djakovic O, Nikolic A, Klun I, Bobic B: Effectiveness of spiramycin in murine models of acute and chronic toxoplasmosis. *Int J Antimicrob Agents* 25, 226–230 (2005)
30. Derouin F, Almadany R, Chau F, Roveux B, Pocidalo JJ: Synergistic activity of azithromycin and pyrimethamine or sulfadiazine in acute experimental toxoplasmosis. *Antimicrob Agents Chemother* 36, 997–1001 (1992)
31. Chang HR, Pechere JC: Activity of spiramycin against *Toxoplasma gondii* *in vitro*, in experimental infections and in human infection. *J Antimicrob Chemother* 22(Suppl B), 87–92 (1988)
32. Chamberland S, Kirst HA, Current WL: Comparative activity of macrolides against *Toxoplasma gondii* demonstrating utility of an *in vitro* microassay. *Antimicrob Agents Chemother* 35, 903–909 (1991)
33. Dumas JL, Chang R, Mermillod B, Piguet PF, Comte R, Pechere JC: Evaluation of the efficacy of prolonged administration of azithromycin in a murine model of chronic toxoplasmosis. *J Antimicrob Chemother* 34, 111–118 (1994)
34. Zenclussen AC: Regulatory T cells in pregnancy. *Springer Semin Immunopathol* 28, 31–39 (2006)
35. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT: Normal human pregnancy is associated with an elevation in the immune suppressive CD25⁺ CD4⁺ regulatory T-cell subset. *Immunology* 112, 38–43 (2004)
36. Eddahri F, Oldenhove G, Denanglaire S, Urbain J, Leo O, Andris F: CD4⁺ CD25⁺ regulatory T cells control the magnitude of T-dependent humoral immune responses to exogenous antigens. *Eur J Immunol* 36, 855–863 (2006)